Myxo-bacteria

The Word is Spreading

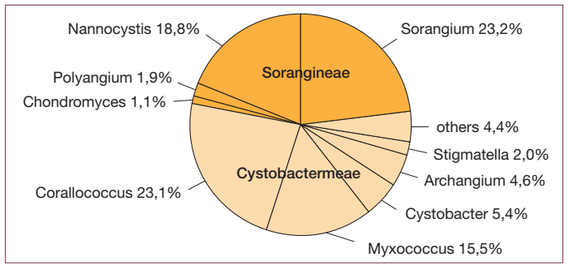
Swarm of Corallococcus

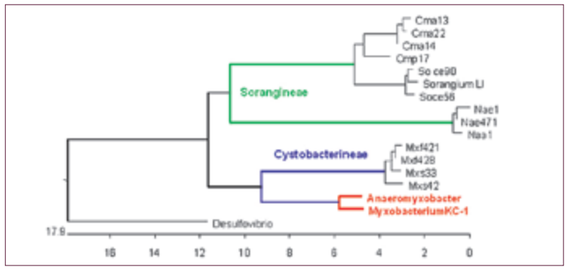
Myxobacteria are gram-negative bacteria that differ from other bacteria in their ability to differentiate into "fruiting bodies." These multicellular structures remind of the real fruiting bodies of lower fungi, with which they are often confused. Together, myxobacteria are known to produce over 500 novel secondary metabolites, making them competitive with established sources of natural products such as the actinomycetes. Both morphological differentiation – a property also common to the actinomycetes – and secondary metabolite biosynthesis require complex regulation. It is therefore not surprising that both groups of bacteria possess gigantic genomes. In fact, the myxobacterium ***Sorangium cellulosum*** has the largest genome yet discovered; at 13.1 million base pairs, its size is comparable to that of higher organisms such as Baker's yeast.

Until the Gesellschaft für Biotechnologische Forschung (GBF) in Braunschweig decided to make the myxobacteria a focus of research, very little was known about this group of organisms. The GBF's (now renamed to Helmholtz Centre for Infection Research (HZI)) research platform has made a decisive contribution to the exploitation of myxobacteria as a source of natural products. Most critically, microbiological know-how was supported by fermentation facilities with capacities of as many as 6,000 liters. This scale of growth supplied sufficient material for the natural product chemists to isolate, purify and elucidate the structures of new metabolites. Furthermore, the compounds were obtained in adequate yields to allow the evaluation of biological activity.

The myxobacterial strain bank at the HZI has continued to expand over the last 25 years. Today, it comprises approximately 7,000 isolates, a diversity not matched anywhere else in the world. Analysis of the strain collection has revealed that the ability to synthesize natural products is not limited to a certain species or even genus of myxobacteria. However, the three suborders of myxobacteria (Cystobacterineae, Sorangiinea and Nannocystineae), tend to exhibit order-specific metabolite profiles. Strikingly, approximately 90% of isolated ***Sorangium*** species produce natural products; together, these compounds comprise 48% of the new biological agents derived from myxobacteria.

Some metabolites are produced by several different strains, while others are relatively rare. Typically, myxobacteria do not biosynthesize a single natural product belonging to a structural type, but often a family of closely-related metabolites. Remarkably, at least 30 different epothilone variants have been isolated from extracts of the epothilone producer. In addition, myxobacteria often produce numerous, structurally distinct secondary metabolites at the same time, a feature shared with the actinomycetes. Due to the economic relevance of epothilone and soraphen, their producing organisms have been studied intensively. Remarkably, producers of both compounds were found in samples obtained from locations worldwide. Thus, the ability to biosynthesize soraphen and epothilone is independent of geographical origin. However, the strains dif-



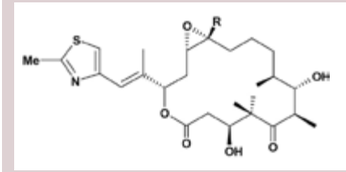


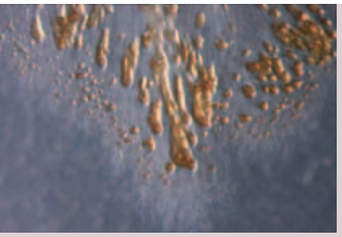
The strain library of the HZI consists of approximately 7.000 myxobacterial isolates.

Differentiation processes
Structural or functional specialization of cells culminating in the formation of fruiting bodies in myxobacteria

Fermenter

Epothilon A: R = H
Epothilon B: R = Me

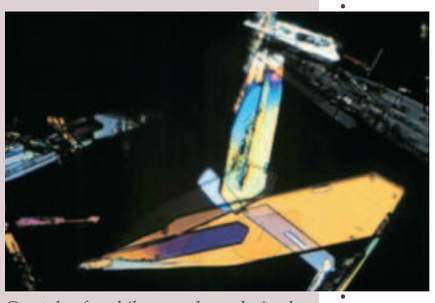




Colony of a halophilic myxobacterium

Microaerophiles
Microorganisms that grow optimally when the oxygen concentration in the medium is considerably lower than that of normal air (approximately 20%).

Carotinoid
Yellow, orange and red lipophilic pigments found in the plant kingdom, which are typically built from eight prenyl units, and thus are classified as class of terpenes.



Crystals of epthilone under polarized light

Ceramide
A simple sphingolipide, a class of lipids found in the cell membrane.

Cerebroside
Sphingolipide from the brain which consists of ceramid and galactose.

Germacrane
Volatile compounds which are often components of essential oils, and which belong to the substance family of the so-called 'sesquiterpenes'.

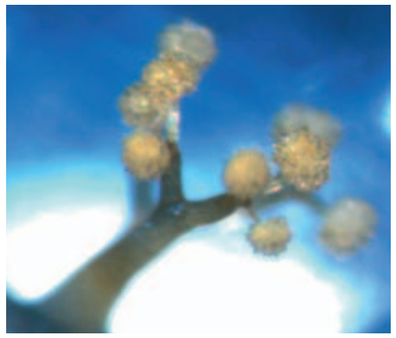
fer with respect to other physiological characteristics, such as the regulation of secondary metabolism.

To date, the myxobacteria have been classified as strictly aerobic, mesophilic organisms, with narrow pH-range and salt tolerance. Recently, however, ***microaerophilic*** and even strictly anaerobic myxobacteria have been isolated. New myxobacteria have also been identified from 'extreme' environments. For example, halophilic and salt-dependent myxobacteria were isolated from marine and saline soil samples obtained from the foreland of the Harz mountains, while alcalophilic strains have been discovered which grow only at pH 9.5. In addition, soil samples from warmer climates yielded thermophilic myxobacteria. These strains exhibit a growth optimum of 42–46 °C, considerably higher than the 30 °C favored by all other myxobacteria. This feature results in considerably faster growth, allowing these myxobacteria to "compete" with other bacteria. DNA sequence comparison reveals that some of the newly-identified myxobacteria occupy completely new branches of the phylogenetic tree. Clearly, our understanding of myxobacterial physiology and metabolism is only in its infancy.

Myxobacteria – producers of surprising structures

The biosynthetic capability of myxobacteria is arguably as striking as their complex life cycles. Some of the metabolites are relatively common in nature. These include ***carotinoids*** (terpenoids), iron transporters, ***ceramides*** and ***cerebrosides***, ***germacranes***, alkaloids (harmane) and geosmin, the last of which is responsible for the strong earthy smell of some samples. Myxobacteria also produce steroids, which although prevalent in nature, are rare in bacteria. Together, myxobacteria generate approximately 100 unique structures, and some 500 variants. Analysis of the structures shows that many of them are complex polyketides, nonribosomally-made peptides, or hybrids of these two compound classes.

Typical myxobacterial secondary metabolites are rarely identical to the products of other microorganisms, although compounds obtained from marine sources show the greatest similarity. For example, the myxobacterial compound thiangazol A differs only slightly from tantazol B, a cytotoxic metabolite from the cyanobacterium ***Scytonema mirabile***. Further similarities are observed with natural products derived from sponges. For example, the polyketide backbone of the myxobacterial depsipeptide chondramides differs by only one carbon from that of the marine metabolites jaspamide, geodiamolide and neosiphoniamolimide; the peptide portions of the structures also display a high degree of correspondence.



Chondromyces crocatus

Although the biological effects of several myxobacterial metabolites remain unknown, the majority of compounds were isolated on the basis of their activities in cell-based assays. Remarkably, approximately 75% of the basic structures that have been isolated from myxobacteria display biological activity, including antibacterial, antifungal and/or cytotoxic properites. The spectrum of activity and mode of action of specific substances are as diverse as their structures. Strikingly, some myxobacterial compounds exhibit biological activity which is rarely observed from other natural products – yet another reason to advance research on myxobacteria.

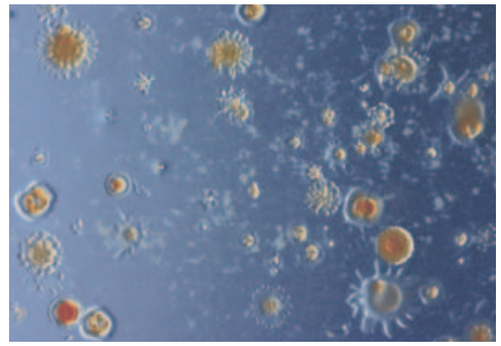
New bioactive natural antibiotics active against bacterial infectious disease

For many years, the prevailing view was that bacterial diseases such as typhoid, meningitis, tuberculosis and pneumonia, could be treated with our existing arsenal of antibiotics. Recently, though, an increasing number of bacteria have developed resistance against well-established antibiotics and, of particular concern, many strains have gained multi-resistance against several different drugs. For some of these bacteria, vancomycin remains one of the few active agents, and is thus considered an 'antibiotic of last resort.' However, bacteria are starting to emerge with resistance even to vancomycin. These circumstances have motivated efforts to identify new antibacterials from myxobacteria.

To date, more than 15 new antibiotic substances have been isolated, comprising different structural types and with disparate modes of action. Sorangicin, corallopyronin, myxopyronin and ripostatin inhibit bacterial ***RNA polymerase***, an enzyme which is targeted by only a few other natural products. Two further antibiotics from myxobacteria, thuggacin and maracin, are characterized by their spectrum of activity, as they are specific for ***mycobacteria***. Notably, all of these compounds exhibit low levels of cytotoxicity to eukaryotic cells, suggesting that their side-effects in humans will be minimal.

New bioactive natural products active against mycoses

A great number of the natural products produced by myxobacteria are antifungal, that is they kill fungi or inhibit their growth; rare and even entirely novel modes of actions have been discovered. Myxothiazol, whose antifungal activity is based on the inhibition of electron transport in the ***mitochondrial/eukaryotic respiratory chain***, has been the subject of particular interest. Myxothiazol, along with stigmatellin from ***Stigmatella aurantiaca***, has been exploited to investigate the bc_1-complex of the mitochondrial respiratory chain. One issue with developing myxothiazol for the clinic, however, is that it also inhibits the respiratory chain in mammals, with overall toxicity determined by drug uptake, distribution and metabolism. Following the elucidation of the chemical structure of myxothiazol in 1978, 20 more electron transport inhibitors of different structural types were found, as well as many variants. The compounds are produced by different genera, with a few strains capable of producing several different inhibitors simultaneously. Interestingly, the β-methoxyacrylate pharmacophor of myoxthiazol is related to that of the strobilurins, a compound class that has been developed into a new, commercially successful class of fungicides used for plant protection. Soraphen is another antifungal secondary metabolite from myxobacteria. It was discovered in 1985 in cultures of ***Sorangium cellulosum*** due to its strong inhibitory effect against a broad range of fungi. The strain had been isolated a few years earlier at the GBF, from goat dung obtained from the island of Djerba, Tunisia. Investigations into the mode of action led to the astonishing result that soraphen exhibits a novel mode of action: it inhibits with high specificity the acetyl-CoA carboxylase of fungi, an enzyme of central significance to fatty acid synthesis. More surprisingly, the acetyl-CoA-carboxylase from higher plants was completely insensitive to the agent, in contrast to the enzyme from rat liver. This finding led to the introduction of soraphen as an industrial fungicide for plant protection. Subsequently, field research demonstrated that the fungus ***Venturia inaequalis***, which is responsible for apple scrab, is completely inhibited by 10 g/ha of soraphen. Infections of Riesling-Silvaner grapes by ***Botrytis cinereae*** were prevented by 25 g/ha soraphen and seet dressing with 30 g/100 kg soraphen inhibited the development of mildew by ***Erysiphe graminis***. The quantities of soraphen required for these studies were obtained by strain and process optimization. In this way, the yield of soraphen was increased from 3 mg/l in the year 1986 to 1.5 g/l by 1990. However, toxicological studies on rats suggested that soraphen acts as a teratogen, and it also provoked allergic reactions from several test animals. These findings brought further development of sorpahen to an end. Nonetheless, soraphen remains a potential lead structure for plant protection, and its novel mode of action is used today as a screening model for new antifungal substances.



Colonies of ***Sorangium cellulosum***

Mitochondrial/eukaryontic respiratory chain
The respiratory chain is located on the inner membrane of mitochondria. It consists of several multienzyme complexes which transmit electrons in a chain reaction from the energy-rich intermediate $NADH^+$ (and $FADH^2$), gradually transferring energy to molecular oxygen. In this process water is created. The resulting energy is used to transport protons from the interior of the mitochondrion to the cytosol, thereby building up an electrochemical gradient. The flow of protons back across the membrane is used by the membrane-localized ATP-synthase for the generation of ATP from ADP and Pi. The biochemical process that takes place in the respiratory chain is called oxidative phosphorylation.

RNA polymerase
An enzyme which transcribes the genetic information encoded in DNA into RNA. RNA molecules called messenger RNA (mRNA) serve as the templates for protein synthesis.

Mycobacteria
A group of Gram-positive bacteria characterized by a high content of waxy lipids on their surface; this feature probably renders them resistant to many adverse environmental conditions. Most mycobacteria are harmless soil inhabitants, but two species cause serious diseases: ***Mycobacterium tuberculosis*** is the causative agent of tuberculosis, a disease that is on the rise because of multiresistant germs, while ***Mycobacterium leprae*** is responsible for leprosy, a disease which infects 5 million people world-wide.

Cytoskeleton
Network built from proteins of fibrous cell structures (filaments) that are dynamically constructed and dismantled. The cytoskeleton is responsible for the mechanical stabilization of the cell, its shape and cell motility.

Microfilaments
Polymeric structures formed from globular, 4 nm large actin units. Two polymer chains coil around each other to form a helix. Along with myosin, microfilaments form a contractile ring that separates one cell into two daughter cells during cell division.

Microtubules
Tiny tubes with a diameter of 25 nm, whose wall is built from 13 protofilaments. The protofilaments are, in turn, composed of tubulin units which are strung together. During cell division, microtubules form a spindle apparatus, which is responsible for the correct distribution of chromosomes to the daughter cells.

Other myxobacterial compounds show antifungal activity through inhibition of protein biosynthesis; substances which hinder the export of proteins from the cell nucleus, change membrane permeability or disrupt osmoregulation. In future, it will be of interest to more fully elucidate the mode-of-action of these and other as yet unidentified myxobacterial compounds, in order to develop more effective treatments for fungal infections. Recently a few substances which were originally discovered for their antifungal acitivity have raised interest as prospective anticancer agents due to their effects on the ***cytoskeleton*** of eukaryotic cells.

New agents against cancer

Although the reason remains unknown, it has become apparent over the last several years that myxobacteria produce numerous natural products that almost exclusively show activity against the cells of higher organisms. Such substances obviously have potential as novel anticancer agents. Even in cases where a natural product is shown to have undesirable side-effects, it can still be exploited as a biochemical tool for investigating modified metabolic pathways in cancer cells.

Some of these compounds interact with the cytoskeleton of higher cells. The eukaryotic cytoskeleton is principally comprised of ***microfilaments*** and ***microtubules***, both polymeric protein structures which are continuously built and dismantled from simpler monomeric units, actin and tubulin, respectively. This dynamic polymerization and depolymerization process, which is intimately linked to cytoskeletal function, is disturbed by myxobacterial natural products, which target either the assembly and deconstruction processes. For example, rhizopodin inhibits the polymerization of actin into microfilaments, whereas chondramide promotes microfilament assembly. Microfilaments are important for both the architecture and movement of cells, but also for the last step in cell division, cytokinesis. Therefore, substances that disrupt actin polymerization inhibit the proliferation of cancer cells, which divide more often than normal cells. Nonetheless, it is currently a matter of debate whether such substances can be introduced as cancer therapeutics, and to date, no such agent is in use.

Microtubules are important for transport within the cell and form the spindle apparatus during mitosis, ensuring that both daughter cells obtain a complete set of chromosomes. When the process of microtubule formation and deconstruction is disturbed, cell division does not take place. Two classes of substances interact with microtubules, and both are on the market as cytostatic agents. Vinblastin and Taxol® (also called paclitaxel) derive from plants, the Madagascar Periwinkle and the Pacific yew, respectively. However, while vinblastin inhibits microtubule assembly, Taxol inhibits the dismantling process. The vinblastin mode-of-action is also exhibited by a myxobacterial compound, tubulysin. Tubulysin, which was isolated from a strain found in a compost heap in a botanical garden in Freiburg, Germany, inhibits the formation of an intact spindle apparatus, thereby inducing cell death. Tubulysin is currently under intensive investigation as a potential anti-cancer agent, with initial promising results.

The treasure from the river Sambesi

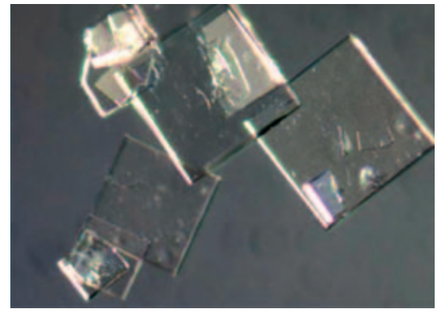
A soil sample obtained from the shore of the river Sambesi yielded a myxobacterial strain that was identified as ***Sorangium cellulosum*** So ce90. The strain harbored a secret treasure – a substance with the same mode of action as Taxol®. At that time, Taxol was being celebrated as an outstanding new development on the cytostatics market; it now boasts sales of more than $1 billion per annum. The new compound, named epothilone, had at least one important advantage relative to Taxol – it was also active against Taxol-resistant tumor cells. Not surprisingly, epothilone was adopted by the pharmaceutical industry as a lead structure for development as an anticancer agent. As a result, several natural and synthetic epothilones are in advanced clinical trials for the treatment of various tumors, including breast and prostate. One analog, ixabepilone, was recently approved by the United States Food and Drug Administration (FDA) for use in the treatment of aggressive metastatic or locally advanced breast cancer, which no longer respond to other chemotherapies.

Riesling-Sylvaner grapes infested with a gray mold.

Vine at the same location that was treated with soraphen.

What does the future hold? The latent potential of the genome

Comparison of the number of natural products known from myxobacteria and actinomycetes with the number of potential secondary metabolite clusters encoded in the genomes reveals that the biosynthetic potential of these microorganisms has been largely unexplored. Although it is clear that the discovery of new strains alone will likely yield novel compounds, full exploitation of the metabolic reservoir of these microbes will depend on bringing the tools of molecular biology and genomics to bear.



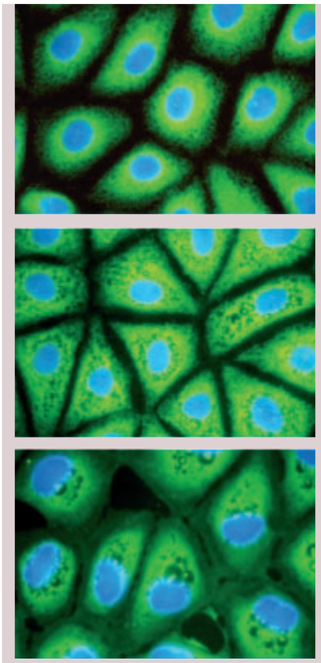
Crystals of tubulysins

Analysis of 16S-rRNA is pivotal to identifying new strains from microbiological samples. This method allows deconvolution of mixed microbial populations, for example those produced during 'metagenomics' experiments, and thus rapid determination of whether myxobacteria or closely-related microorganisms are present. One target of such analysis has been marine sponges, which have already yielded many potent, structurally complex natural products. These compounds are typically produced in low yields, a feature which has so far limited the wide-spread exploitation of these agents in the clinic. The fact that terrestrial microbes such as myxobacteria produce similar structures to those derived from sponges (chondramide and jaspamide are one notable example), suggests that the true producers in the marine organism are microbial (so called 'endosymbionts'). Therefore one potential way to deal with the issue of metabolite yield would be to identify the genes responsible for a particular metabolite, and to transfer them to an alternative host for compound production. Clues as to what to look for in the metagenomic 'gene bank' of the sponge can be obtained by inspection of the terrestrial counterpart. (How the further advancement on the path to new natural products and new production lines could look like can be read about in the chapter "Adventure in the metagenome" starting page 76.)

Molecular Biology and Genomics. Molecular biology not only provides tools for the isolation of new strains, but also contributes to improving the production of specific bioactive metabolites from known bacteria. A good test case for this technology will be the recently-sequenced genome of the model myxobacterium ***Sorangium cellulosum*** So ce56. The genome, sequenced under the auspices of the so-called GenoMik-Network, is the largest yet identified in a bacterium. In fact, ***S. cellulosum*** has as many genes as the fruit fly – a full one-third of those present in humans. It is likely that much of the genome supports the complex life cycle of the myxobacterium, but a large portion is also dedicated to secondary metabolism. Analysis of the gene content suggests that the strain has the potential to produce more than twenty natural products, only three of which are currently known. It is clear that much is likely to be gained by applying molecular biology methods to awaken the latent genome of So ce56, a field called 'systems biology'.

On the path to systems biology

Systems biology (in this context often called "genome mining") aims to arouse the dormant secondary metabolite potential of myxobacteria, by, for example, activating the genes involved in natural product biosynthesis. To do this, however, requires a comprehensive understanding of the underlying regulatory networks. Such networks can be analyzed by whole-genome based approaches, including transcriptomics, proteomics and metabolomics. Insights obtained by such methods can then be applied to newly-sequenced model strains using bioinformatics. As more myxobacterial genomes



V-ATP-inhibitors lead to characteristic changes in the inner structure of cells, shown here by a green coloring of the protein which is situated in the membrane of the endoplasmatic recticulum. (Picture on top: normal cells). In the presence of arachazolid (middle picture) or apicularen (lower picture). Vacuole-like structures are formed in which protein-precipitation is visible. The nuclei are colored blue.

16S-rRNA-(gene-)analysis
A method for the determination of the genetic relationships between microorganisms. The 16S-rRNA gene sequence serves a marker for relatedness.

Metagenome bank/ Metagenome library
Collection of clones that carry different sections of a metagenome as foreign DNA.

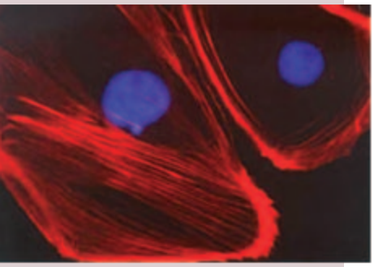
Biosynthesis module
Subunits of complex biosynthetic enzymes.

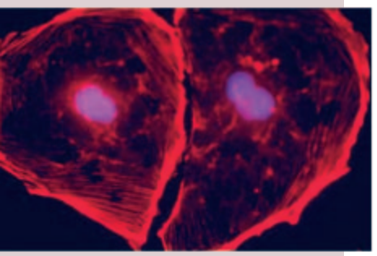
are sequenced, it should become possible to discover novel secondary metabolites, strictly on the basis of comparative analysis with other strains. If we can understand regulatory processes, we will not only be able to activate the production of unknown compounds, but to increase the yield of recognized metabolites, allowing for more economic fermentation. Such analyses should identify bottlenecks in production, which can then be targeted for modification by genetic engineering.

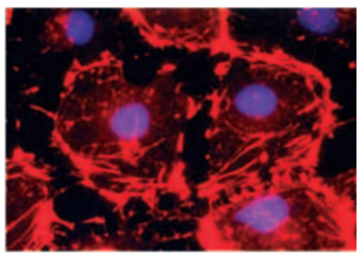
Myxobacterial agents from *E. coli* cultures?

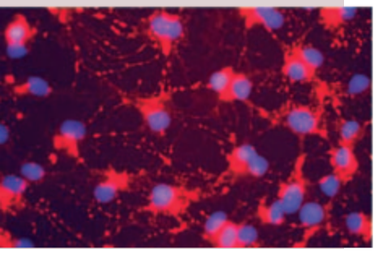
Production of recombinant proteins in easily cultured host organisms such as ***Escherichia coli*** has long been the state-of-the-art in biotechnology. It is much more difficult, however, to transfer entire, complex biosynthetic pathways to such heterologous hosts. Nonetheless, such advances are likely to occur in future.

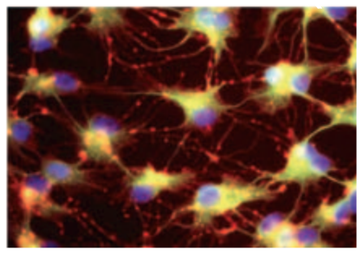
The following are important considerations in the choice of the host bacterium for a myxobacterial biosynthetic gene cluster. The host should exhibit rapid growth, be amenable to genetic modification, and grow efficiently under fermentation conditions. It should be able to produce all of the precursors which are required for secondary metabolite biosynthesis. In theory, a fast-growing myxobacterium could be adapted as a heterolgous hosts, and encouragingly, strain isolation efforts have already yielded thermophilic myxobacteria which exhibit short generation times. However, other secondary metabolite producers such as the actinomycetes and pseudomonads, and perhaps even ***Escherischia coli***, may ultimately be suitable. As proof-of-principle, we recently described the production of the myxobacterial metabolites such as epothilone, myxothiazol and myxochromide in ***Pseudomonas putida*** and ***M. xanthus***. Such host bacteria may also be suitable for attempts to unlock the genetic potential of metagenomic DNA obtained from marine samples. Further developments in this area of research are eagerly anticipated.











Rhizopodin perturbs the composition of microfilaments in the cell. The microfilaments are colored red, while the cell nuclei are colored blue (above, picture of a normal cell). Following 2 h of treatment, gaps in the actin filaments are visible (middle left), while 4 h post-treatment, only short pieces remain (middle right). After one day, only small lumps of actin around the cell nucleus and in the fibrous cellular extensions remain (below left). In the figure below right, the microtubules are colored green.

Mixed structures – biologically recombined

Combinatorial biosynthesis – the 'mixing and matching' of biosynthetic genes both within and between organisms – has the potential to generate a large number of 'hybrid' natural products. But to truly establish this technology, we will need to understand in much greater detail how the biosynthesis of secondary metabolites occurs on a molecular level. Although significant advances have been made, much remains to be done. In the meantime, it is clear that myxobacteria are an important source of ***biosynthetic 'modules'*** that can be exploited in such experiments. Not only can portions of myxobacterial metabolites be intermixed, but they should be amenable to combination with structures derived from actinomycetes and other bacteria. As such experiments have very recently met with success in the laboratory, the future of myxobacterial secondary metabolite research looks bright.

Klaus Gerth, Herbert Irschik, Rolf Jansen,
Brigitte Kunze, Rolf Müller, Florenz Sasse

Additional Literature

Gerth K, Pradella S, Perlova O, Beyer S, Müller R: Myxobacteria: proficient producers of novel natural products with various biological activities - past and future biotechnological aspects with the focus on the genus Sorangium. J Biotechnol 2003, 106:233-253

Bode HB, Müller R: The impact of bacterial genomics on natural product research. Angew Chem Int Ed 2005, 44:6828-6846

Reichenbach H, Höfle G.: Myxobacteria as producers of secondary metabolites. In Drug Discovery from Nature, eds. Grabley, S. & Thiericke, R. Berlin: Springer; 1999:149-179.

Wenzel SC, Müller R: Myxobacterial natural product assembly lines: fascinating examples of curious biochemistry. Nat Prod Rep 2007, 24:1211-1224

Bode HB, Müller R: Analysis of myxobacterial secondary metabolism goes molecular. J Ind Microbiol Biotechnol 2006, 33:577-588

Altmann K-H, Höfle G, Müller R, Mulzer J, Prantz K: (2009) The Epothilones - An Outstanding Family of Anti-Tumour Agents: From Soil to the Clinic (Springer, New York)

Weissmann KJ, Müller R*: Myxobacterial secondary metabolites: Bioactivities and modes-of-action. Natural Product Reports 2010, 27(9):1276, E-page: 1295

Wenzel SC, Müller R: The impact of genomics on the exploitation of the myxobacterial secondary metabolome. Nat Prod Rep 2009, 26(11): 1385, E-page: 1407, PubMed

Links on the Web

DECHEMA Gesellschaft für chemische Technik und Biotechnologie e.V.
www.dechema.de

Institut für Pharmazeutische Biotechnologie an der Univeristät des Saarlandes
www.myxo.uni-saarland.de

Helmholtz-Zentrum für Infektionsforschung
www.helmholtz-hzi.de

Helmholtz-Institut für Pharmazeutische Forschung Saarland
www.helmholtz-hzi.de/hips

Myxobacteria are a rich source of natural products; an extract is shown on the left and the pure substance on the right.